CELL-BASED RNA INTERFERENCE AND RELATED METHODS AND COMPOSITIONS

RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. Provisional Application 60/414,605, filed Sept. 27, 2002 and entitled "Methods for generating genetic 'knock-outs' using RNA interference in stem cells", which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERAL FUNDING

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BACKGROUND

"RNA interference", "post-transcriptional gene silencing", "quelling" — these different names describe similar effects that result from the overexpression or misexpression of transgenes, or from the deliberate introduction of double-stranded RNA into cells (reviewed in Fire A (1999) Trends Genet 15: 358–363; Sharp PA (1999) Genes Dev 13: 139–141; Hunter C (1999) Curr Biol 9: R440–R442; Baulcombe DC (1999) Curr Biol 9: R599–R601; Vaucheret et al. (1998) Plant J 16: 651–659). The injection of double-stranded RNA into the nematode *Caenorhabditis elegans*, for example, acts systemically to cause the post-transcriptional depletion of the homologous endogenous RNA (Fire et al. (1998) Nature 391: 806–811; and Montgomery et al. (1998) PNAS 95: 15502–15507). RNA interference, commonly referred to as RNAi, offers a way of specifically and potently inactivating a cloned gene, and is proving a powerful tool for investigating gene function.

Significant breakthroughs in RNAi technology have permitted the application of this technique to the cells of higher eukaryotes, including humans and

other mammals. However, RNAi techniques have not been used to stably transfect mitotically active cells, such as stem cells, tumor cells or certain differentiated cells, in a manner that permits the reconstitution of tissues, organs and whole organisms that comprise cells affected by an RNAi construct.

The invention is intended to address these and other shortcomings in the field of RNAi technology.

SUMMARY OF THE INVENTION

In certain aspects, the invention provides systems which use RNA interference to stably and specifically target and decrease the expression of one or more target genes in cells, such that the cells may be introduced into a living organism and propagated without significant loss of the RNA interference effect. In certain aspects the invention provides methods for modifying cells ex vivo with a short hairpin RNA (shRNA) expression construct to achieve an RNA interference effect and introducing the cells into a subject. In certain aspects the invention provides vectors and methods for controlling the temporal and spatial expression of a shRNA construct in cells and organisms.

In one aspect, the invention provides methods for introducing into a subject a population of stem cells having partial or complete loss of function of a target gene, the method comprising: a) introducing a nucleic acid construct encoding an shRNA into stem cells to generate transfected stem cells, wherein the shRNA is complementary to a portion of the target gene; and b) introducing the transfected stem cells into the subject, wherein the transfected stem cells propagate within the subject and retain partial to complete loss of function of the target gene. Optionally, the target gene participates in a disease process in the subject. The transfected cells may replace a population of diseased cells in the subject; the diseased cells may be ablated prior to administration of the cells. In certain embodiments, the shRNA construct is expressed constitutively. In other embodiments, shRNA construct expression is conditional. For example, expression of the shRNA may conditional on the presence or absence of a substance administered to the subject. shRNA expression may be cell lineage specific, either because the shRNA expression is

driven by a lineage specific promoter or because introduction of the shRNA construct is limited to cells of a particular lineage. Optionally, the cells are stem cells, such as hematopoietic stem cells or embryonic stem cells. In certain embodiments, the transfected stem cells are cultured so as to generate a population of further differentiated transfected stem cells for introduction into the subject.

In certain aspects the invention provides vectors for stably or controllably introducing shRNA constructs into cells. Such vectors may be retroviral vectors, such as lentiviral vectors.

In certain aspects, the invention provides methods for introducing into a subject a population of differentiated cells having partial or complete loss of function of a target gene, the method comprising: a) introducing a nucleic acid construct encoding an shRNA into stem cells to generate transfected stem cells, wherein the shRNA is complementary to a portion of the target gene; b) culturing the transfected stem cells to generate transfected differentiated cells having partial or complete loss of function of a target gene; and c) introducing the transfected differentiated cells into the subject, wherein the transfected differentiated cells retain partial to complete loss of function of the target gene.

In certain aspects, the invention provides methods of treating a disease associated with the expression of a target gene in a population of cells, the method comprising: a) introducing a nucleic acid construct encoding an shRNA into stem cells to generate transfected stem cells, wherein the shRNA is complementary to a portion of the target gene; and b) introducing the transfected stem cells into the subject,

In further aspects, the invention provides non-human mammals comprising a population of stem cells comprising a nucleic acid construct encoding an shRNA, or progeny cells thereof, wherein the cells exhibit partial to complete loss of function of a target gene.

In one aspect, the invention provides compositions formulated for administration to a human patient, the composition comprising: a) a stem cell comprising a nucleic acid construct encoding an shRNA, wherein the shRNA is complementary to at least a portion of a target gene, and wherein the cells exhibit

partial to complete loss of function of a target gene; and b) a pharmaceutically acceptable excipient.

In certain aspects, the invention provides methods for identifying a gene that affects the sensitivity of tumor cells to a chemotherapeutic agent, the method comprising: a) introducing into a subject a transfected stem cell comprising a nucleic acid construct encoding an shRNA, wherein the shRNA is complementary to at least a portion of a target gene, wherein the transfected stem cell exhibits decreased expression of the target gene, and wherein the transfected stem cell gives rise to a transfected tumor cell in vivo; b) evaluating the effect of the chemotherapeutic agent on the transfected tumor cell. Optionally, evaluating the effect of the chemotherapeutic agent on the transfected tumor cell comprises: administering the chemotherapeutic agent to the subject and measuring the quantity of tumor cells derived from the transfected stem cell. A method may further comprise comparing the quantity of tumor cells derived from the transfected stem cell to the quantity of tumor cells derived from the transfected stem cell in a control subject that has not received the chemotherapeutic agent.

In certain aspects, the invention provides methods for identifying a gene that affects the sensitivity of tumor cells to a chemotherapeutic agent, the method comprising: a) introducing into a subject a plurality of transfected stem cells, wherein each transfected stem cell comprises a nucleic acid construct comprising a representative shRNA of an shRNA library, and wherein a representative shRNA of an shRNA library is complementary to at least a portion of a representative target gene, wherein a plurality of the transfected stem cells exhibits decreased expression of a representative target gene, and wherein a plurality of the transfected stem cells gives rise to transfected tumor cells in vivo; b) administering a chemotherapeutic agent; and c) identifying representative shRNAs that are enriched or depleted by treatment with the therapeutic agent. In a further aspect the invention provides a method of administering a chemotherapeutic agent to a patient, the method comprising: a) administering the chemotherapeutic agent; and b) administering a nucleic acid that causes RNA interference of a gene that is associated with chemotherapeutic resistance.

In certain aspects, the invention provides a barcoded shRNA library comprising a plurality of representative shRNAs, wherein the majority of representative shRNAs are associated with a barcode tag. Optionally, the representative shRNAs are partially complementary to representative genes, and wherein a majority of representative gene are known or suspected to be involved in a cancer.

In certain aspects, the invention provides methods of determining a function of a gene comprising: introducing small hairpin RNA which targets mRNA of the gene into cells; maintaining the cells under conditions in which the small hairpin RNA is stably expressed and RNA interference of the mRNA occurs; introducing the cells into a non-human mammal, thereby producing a knockout non-human mammal; and assessing the phenotype of the knock-out non-human mammal compared to a control mammal, thereby identifying a function of the gene. In some embodiments, a the invention provides a method of determining the contribution of a gene to a condition comprising: a) introducing small hairpin RNA which vary in their ability to inactivate mRNA of the gene into cells, thereby producing a panel of a discrete set of cells in which the mRNA of the gene is inactivated to varying degrees in each set of cells; b) maintaining the cells under conditions in which the small hairpin RNA is stably expressed and RNA interference of the mRNA occurs; c) introducing each set of cells into a separate non-human mammal, thereby producing a panel of knockout non-human mammals in which the mRNA of the gene is inactivated to varying degrees in each non-human mammal; and d) assessing the phenotype of each knock-out non-human mammal compared to a control mammal, thereby determining the contribution of the gene to the condition.

In certain aspects the invention provides a method of engineering cells ex vivo so that the cells exhibit reduced expression of a gene product comprising: a) removing cells from a host; and b) introducing a construct encoding a small hairpin RNA into the cells such that the small RNA is stably expressed and induces RNA interference of the gene product.

In certain aspects the invention relates to the discovery that a cell expressing a shRNA construct may retain a stable RNA interference effect even after excision

or other inactivation of the shRNA construct. In certain embodiments, the invention provides a method for introducing into a subject a population of stem cells having partial or complete loss of function of a target gene, the method comprising: a) introducing a nucleic acid construct encoding an shRNA into stem cells to generate transfected stem cells, wherein the shRNA is complementary to a portion of the target gene, such that expression of the target gene is decreased; b) removing or inactivating the nucleic acid construct; c) verifying that expression of the target gene remains decreased; d) introducing the stem cells into a subject, wherein the stem cells propagate within the subject and retain partial to complete loss of function of the target gene. Optionally, the nucleic acid construct comprises a lox site and removing or inactivating the nucleic acid construct comprises introducing or activating Cre.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram showing the process of generation of shRNA expressing lymphomas.

Figure 2 is a schematic diagram showing the retroviral construct design for p53-A, p53-B and p53-C. p53-A has an MMLV retroviral backbone, while p53-B and p53-C are derived from MSCV.

Figure 3 is a diagram showing the approximate location of the hairpin sequence on the p53 cDNA.

Figure 4 is a diagram showing the PCR amplification of tumor and control DNA with shRNA-specific primers. Both tumors show the presence of the hairpin construct, while control pre-infection stem cells do not.

Figure 5 is a diagram showing survival curves for mice injected with stem cells infected with either Control or p53 shRNA constructs.

Figures 6A-6C are H&E slides of a lymphoma (Figure 6A), a lung (Figure 6B) and a spleen (Figure 6C) from a mouse with shp53-induced tumors. Lymphoma pathology and aggressive lung and spleen metastasis resemble that seen in p53-/- tumors.

Figure 6D is a TUNEL staining showing only low levels of apoptosis in shp53-induced lymphomas, a characteristic of p53-/- tumors.

Figure 7 is a Western analysis for p53 levels in Murine Embryo Fibroblasts (MEFs) infected with various hairpins targeting p53. Cells were treated with 0.5 ug/ml adriamycin for 6 hours to induce p53 levels. All p53 shRNAs show a reduction in p53 induction, while a GFP shRNA had no effect on p53 levels. Tubulin controls were provided to confirm equal amounts of total protein in each lane.

Figure 8 is a PCR reaction, designed to amplify both the WT and KO p53 allele, and shows the maintenance of the WT allele in a tumor expressing a p53 shRNA. An MSCV control shows loss of the WT allele, while a bcl-2 control shows retention of the WT allele.

Figure 9: Heritable repression of Neil1 expression by RNAi in several tissues. (a) Expression of Neil1 mRNA in the livers of three mice containing the Neil1 shRNA transgene (shRNA-positive) or three siblings lacking the transgene (shRNA-negative) was assayed by RT-PCR (top row is Neil1). An RT-PCR of β-actin was done to ensure that equal quantities of mRNAs were tested for each mouse (second row). Expression of the neomycin resistance gene (neo), carried on the shRNA vector, was tested similarly (third row). Finally, the mice were genotyped using genomic DNA that was PCR-amplified with vector-specific primers (bottom row). (b) Similar studies were performed in the heart. (c) Similar studies were performed in the spleen. Animal procedures have been approved by the SUNY, Stony Brook Institutional Animal Care and Use Committee (IACUC).

Figure 10: Reduction in Neil1 protein correlates with the presence of siRNAs. (a) Expression of Neil1 protein was examined in protein extracts from the livers of mice carrying the shRNA transgene (shRNA-positive) or siblings lacking the transgene (shRNA-negative) by western blotting with Neil1-specific antiserum. A western blot for PCNA was used to standardize loading. (b) The presence of siRNAs in RNA derived from the livers of transgenic mice as assayed by northern blotting using a 300 nt probe, part of which was complementary to the shRNA

sequence. Applicants note siRNAs only in mice transgenic for the shRNA expression cassette.

Figure 11. A. Graph showing a shorter lymphoma onset time Bim or Puma shRNA mice. B, C. Bim and Puma expression are decreased in tumor cells by targeted shRNA.

Figure 12. Survival of tumor cells carrying Bim shRNA as compared to control tumors, during treatment with adriamycin.

Figure 13. Diagram of shRNA screening assay to identify tumor sensitizing shRNAs.

Figure 14. FACS analysis of GFP containing cells in pre-treatment and relapsed tumors.

Figure 15. A. A diagram of a Self-Inactivating retroviral vector (SIN vector) for use with shRNA. B. Demonstration of effectiveness of SIN vector and standard vector in RNA interference.

Figure 16. Southern blot analysis of proviral transgene insertions in the p53C shRNA founder mice. Transgenic founders #3, #8, and #10 have a single proviral insertions site, while the rest of the mice were non-transgenic.

Figure 17. Western analysis of p53 in dermal fibroblasts of p53C shRNA lentiviral transgenic mice (#'s 3, 8, and 10) and non-transgenic littermate controls (#'s 1 and 2), treated with 0.5 ug adriamycin per ml for approximately 6 hours. Lanes 1 and 2 are MEFs infected with either MSCV or p53C shRNA and treated with adriamycin.

Figure 18. Colony formation assay using dermal fibroblasts cultured from lentiviral-mediated p53C shRNA transgenic mice and non-transgenic littermate control. Cells were plated at the indicated cell numbers, and allowed to grow for approximately 3 weeks.

Figure 19. A. Schematic representation of the screening process using population approaches in which biological stimuli are applied to populations of cells containing barcoded shRNAs. B. Images of arrays in the Cy3 and Cy5 channels of

a self-self library hybridization. C. A log-log plot of intensities in Cy3 and Cy5 channels.

Figure 20. A diagram of a methodology for identifying genes that participate in chemotherapeutic resistance and sensitivity.

Figure 21. Cells were infected with RCAS shp53C or a control vector, selected with puromycin for 3 days, and subsequently plated at 25,000 cells per well. Cells were treated with 0.5 ug/ml adriamycin to induce p53.

Figure 22. Cells were infected with either RCAS shp53C or control vector, selected with puromycin for 3 days, and subsequently plated at the indicated cell numbers per well and allowed to grow for approximately 2wks. Data reveal enhanced cell growth for cells expressing RCAS shp53C.

Figure 23. Diagram of site specific shRNA insertion system.

Figure 24. Suppression of luc activity in cells expressing luc shRNAs. Luciferace activity in the shRNA expressing cells is shown relative to cells not expressing shRNA.

Figure 25. A. Excisable shRNA expression vector harboring tamoxifen-regulated cre. B. Wild type MEFS were infected with the Cre-loxP-U6p53CshRNA-PIG virus, and these cells show stable suppression of p53 expression by Western blot.

Figure 26. Addition of 0.5 μM 4-hydroxytamoxifen (4OHT) to cultured cells infected with MSCV CreER/loxP U6p53C PIG virus results in deletion of the provirus from the genome, as measured by Southern blot using a probe that hybridizes to the GFP cassette in the provirus (A). As expected, 4OHT treatment and excision of the provirus also leads to loss of GFP expression, as measured by Western blot (B) or FACS (C).

Figure 27. MSCV Cre/loxP U6p53C PIG in cultured mouse embryonic fibroblasts. Control cells are in the upper panels. Lower panels are tamoxifen treatment panels.

Figure 28. A diagram of a second generation vector.

Figure 29. Western blot showing p53 protein levels in cultured murine embryonic fibroblast cells infected with MSCV Cre/loxP U6p53C PIG or a control vector (MSCV PIG). Virally infected, puromycin selected cells were cultured for 6 days, treated with 0.5uM OHT or vehicle for 24 h, then cultured for a further 6 days. Immediately before harvesting, cells were treated as indicated for 4 h with 0.5 ug/mL adriamycin (ADR), a DNA damaging agent that causes massive induction of p53 in control (MSCV PIG) infected cells. Minimal p53 induction is observed in MSCV Cre/loxP U6p53C PIG infected cells, even 6 days after OHT treatment.

DETAILED DESCRIPTION OF THE INVENTION

1. Overview

In certain aspects, the invention provides systems which use RNA interference to stably and specifically target and decrease the expression of one or more target genes in cells. Recent work has shown that the RNA interference effects of exogenously provided dsRNAs can be recapitulated in mammalian cells by the expression of single RNA molecules which fold into stable "hairpin" structures (Paddison et al. Genes Dev 16(8):948-58 (2002)). Transient transfection of plasmids encoding small "hairpin" RNAs (shRNAs) can achieve a near complete reduction in the levels of a specific protein in a cell. Applicants have now demonstrated that shRNAs can be stably introduced into mammalian cells, introduced into a living organism and propagated without significant loss of the RNA interference effect. A variety of experiments substantiating the discovery are presented in detail in the Examples below. To summarize one such experiment, shRNAs targeted to p53 were introduced into mouse stem cells in culture and transplanted into mice. Applicants have detected the presence of shRNAs in transplanted cells over three months after transplantation. Cells manipulated according to the disclosed methodology may be introduced into a mammal (or used to generate a mammal) and propagated in vivo without significant loss of the RNA interference effects in the cells or their progeny. In certain embodiments, the system takes advantage of gene transfer of DNA or RNA constructs encoding short hairpin RNAs into cells.

Accordingly, in certain aspects, the invention provides systems for reducing the expression of genes (e.g., "knock-out" or partial reduction) in an in vivo model and analyzing the results in a rapid manner. This technology potentially bypasses both the developmental issues of embryonic lethality and compensation seen in traditional "knock-out" mouse systems. RNA inhibition has previously been used to suppress gene expression in mammalian cells in vitro. These groups have also transplanted these cultured cells as xenografts into nude mice. However, the experiments described in this document are the first to stably express shRNAs in stem cells and subsequently use those stem cells to reconstitute a fully functional organ with a targeted gene "knock-out".

Applicants have further discovered a wide range of technological and therapeutic applications for implantable stem cells transfected with stable RNAi constructs.

In certain aspects, methods disclosed herein may be used for ex vivo stem cell therapies. For example, an autologous or heterologous stem cell population may be transfected with a stable RNA interference construct and introduced into a patient, where the modified cells perform a therapeutic function. It is important to note that RNA interference may be used to cause both decreased (e.g., direct RNA interference) or increased expression of genes (e.g., indirect effect). For example, although RNA interference will decrease the expression of a target gene, the target gene itself may be a negative regulator, and therefore the RNA interference will indirectly cause increased expression of the negative regulator.

In further aspects, methods disclosed herein may be used to assess the positive or negative effects of a RNAi on an in vivo process. For example, as described in the examples below, stem cells transfected with a stable shRNA construct may be used to identify gene that contribute to chemotherapeutic sensitivity or resistance in tumor cells. In certain embodiments, such screening methods may be performed in a high throughput format.

2. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article, unless context clearly indicates otherwise. By way of example, "an element" means one element or more than one element.

The term "adult stem cell" is used herein to refer to a stem cell obtained from any non-embryonic tissue. For example, cells derived from fetal tissue and amniotic or placental tissue are included in the term adult stem cell. Cells of these types tend to have properties more similar to cells derived from adult animals than to cells derived from embryonic tissue, and accordingly, for the purposes of this application stem cells may be sorted into two categories: "embryonic" and "adult" (or, equivalently, "non-embryonic").

The term "culturing" includes exposing cells to any condition. While "culturing" cells is often intended to promote growth of one or more cells, "culturing" cells need not promote or result in any cell growth, and the condition may even be lethal to a substantial portion of the cells.

A later cell is "derived" from an earlier cell if the later cell is descended from the earlier cell through one or more cell divisions. Where a cell culture is initiated with one or more initial cells, it may be inferred that cells growing up in the culture, even after one or more changes in culture condition, are derived from the initial cells. A later cell may still be considered derived from an earlier cell even if there has been an intervening genetic manipulation.

The term "including" is used herein to mean, and is used interchangeably with, the phrase "including but not limited to".

The term "or" is used herein to mean, and is used interchangeably with, the term "and/or", unless context clearly indicates otherwise.

A "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal, preferably a mammal.

The term "percent identical" refers to sequence identity between two amino acid sequences or between two nucleotide sequences. Percent identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. Expression as a percentage of identity refers to a function of the number of identical amino acids or nucleic acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST, or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences can be determined by the GCG program with a gap weight of 1, e.g., each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences.

Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, California, USA. Preferably, an alignment program that permits gaps in the sequence is utilized to align the sequences. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. An alternative search strategy uses MPSRCH software, which runs on a MASPAR computer. MPSRCH uses a Smith-Waterman algorithm to score sequences on a massively parallel computer. This approach improves ability to pick up distantly related matches, and is especially tolerant of small gaps and nucleotide sequence errors. Nucleic acid-encoded amino acid sequences can be used to search both protein and DNA databases.

"Stem cell" describes cells which are able to regenerate themselves and also to give rise to progenitor cells which ultimately will generate cells developmentally restricted to specific lineages.

3. Hairpin RNAi Constructs, Vectors and Cells

Many embodiments of the invention employ single-stranded RNA molecules containing an inverted repeat region that causes the RNA to self-hybridize, forming a hairpin structure. shRNA molecules of this type may be encoded in RNA or DNA vectors. The term "encoded" is used to indicate that the vector, when acted upon by an appropriate enzyme, such as an RNA polymerase, will give rise to the desired shRNA molecules (although additional processing enzymes may also be involved in producing the encoded shRNA molecules). As described herein, vectors comprising one or more encoded shRNAs may be transfected into cells ex vivo, and the cells may be introduced into mammals. The expression of shRNAs may be constitutive or regulated in a desired manner. Other technologies for achieving RNA interference in vivo were unreliable; certain constructs were expressible in stem cells but not in differentiated cells, or vice versa. Technology described herein makes it possible to achieve either constitutive or highly regulated expression of shRNAs in vivo across the spectrum of cell types, thereby permitting tightly controlled regulation of target genes in vivo.

A double-stranded structure of an shRNA is formed by a single self-complementary RNA strand. RNA duplex formation may be initiated either inside or outside the cell. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. shRNA constructs containing a nucleotide sequence identical to a portion, of either coding or non-coding sequence, of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Because 100% sequence identity between the RNA and the target gene is not required to practice the present invention, the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain

polymorphism, or evolutionary divergence. Sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50 °C or 70 °C hybridization for 12-16 hours; followed by washing). In certain preferred embodiments, the length of the duplex-forming portion of an shRNA is at least 20, 21 or 22 nucleotides in length, e.g., corresponding in size to RNA products produced by Dicer-dependent cleavage. In certain embodiments, the shRNA construct is at least 25, 50, 100, 200, 300 or 400 bases in length. In certain embodiments, the shRNA construct is 400-800 bases in length. shRNA constructs are highly tolerant of variation in loop sequence and loop size.

An endogenous RNA polymerase of the cell may mediate transcription of an shRNA encoded in a nucleic acid construct. The shRNA construct may also be synthesized by a bacteriophage RNA polymerase (e.g., T3, T7, SP6) that is expressed in the cell. In preferred embodiments, expression of an shRNA is regulated by an RNA polymerase III promoters; such promoters are known to produce efficient silencing. While essentially any PolIII promoters may be used, desirable examples include the human U6 snRNA promoter, the mouse U6 snRNA promoter, the human and mouse H1 RNA promoter and the human tRNA-val promoter. A U6 snRNA leader sequence may be appended to the primary transcript; such leader sequences tend to increase the efficiency of sub-optimal shRNAs while generally having little or no effect on efficient shRNAs. For transcription from a transgene in vivo, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to regulate expression of the shRNA strand (or strands). Inhibition may be controlled by specific transcription in

an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. The use and production of an expression construct are known in the art (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein).

In a preferred embodiment, a shRNA construct is designed with 29 bp helices following a U6 snRNA leader sequence with the transcript being produced by the human U6 snRNA promoter. This transcription unit may be delivered via a Murine Stem Cell Virus (MSCV) -based retrovirus, with the expression cassette inserted downstream of the packaging signal. Further information on the optimization of shRNA constructs may be found, for example, in the following references: Paddison, P.J., A.A. Caudy, and G.J. Hannon, Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci U S A, 2002. 99(3): p. 1443-8; 13. Brummelkamp, T.R., R. Bernards, and R. Agami, A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. Science, 2002. 21: p. 21; Kawasaki, H. and K. Taira, Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Nucleic Acids Res, 2003. 31(2): p. 700-7; Lee, N.S., et al., Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat Biotechnol, 2002. 20(5): p. 500-5; Miyagishi, M. and K. Taira, U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. Nat Biotechnol, 2002. 20(5): p. 497-500; Paul, C.P., et al., Effective expression of small interfering RNA in human cells. Nat Biotechnol, 2002. 20(5): p. 505-8.

An shRNA will generally be designed to have partial or complete complementarity with one or more target genes (i.e., complementarity with one or more transcripts of one or more target genes). The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene, the nature of the shRNA and the level of expression of shRNA (e.g.

depending on copy number, promoter strength) the procedure may provide partial or complete loss of function for the target gene. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

"Inhibition of gene expression" refers to the absence or observable decrease in the level of protein and/or mRNA product from a target gene. "Specificity" refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

As disclosed herein, the present invention is not limited to any type of target gene or nucleotide sequence. The following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Writ family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABLI, BCLI, BCL2, BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, EBRB2, ETSI, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCLI, MYCN, NRAS, PIM 1, PML, RET, SRC, TALI, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF 1, NF2, RB 1, P53, BIM, PUMA and WTI); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

Promoters/enhancers which may be used to control the expression of a shRNA construct *in vivo* include, but are not limited to, the PolIII human or murine U6 and H1 systems, the cytomegalovirus (CMV) promoter/enhancer, the human β-actin promoter, the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR), the SV40 early or late region promoter, the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer, and the herpes simplex virus LAT promoter. Transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained

from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems. Inducible systems, such as Tet promoters may be employed. In addition, recombinase systems, such as Cre/lox may be used to allow excision of shRNA constructs at desired times. The Cre may be responsive (transcriptionally or post-transcriptionally) to an external signal, such as tamoxifen.

In certain embodiments, a vector system for introducing shRNA constructs into cells are retroviral vector systems, such as lentiviral vector systems. Lentiviral systems permit the delivery and expression of shRNA constructs to both dividing and non-dividing cell populations in vitro and in vivo. Examples of Lentiviral vectors are those based on HIV, FIV and EIAV. See, e.g., Lois, C., et al., Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science, 2002. 295(5556): p. 868-72. Most viral systems contain cis-acting elements necessary for packaging, while trans-acting factors are supplied by a separate plasmid that is co-transfected with the vector into a packaging cell line. In certain embodiments, a highly transfectable 293 cell line may be used for packaging vectors, and viruses may be pseudotyped with a VSV-G envelope glycoprotein for enhanced stability and to provide broad host range for infection. In certain aspects, the invention provides novel vectors adapted for use with shRNA expression cassettes. For example, a Gateway recipient sequence may be inserted downstream of the packaging signal to facilitate movement of the shRNA construct to and from different vector backbones by simple recombination. As another example, recombination signals may be inserted to facilitate in vivo transfer of shRNAs from, e.g., a genome-wide shRNA library.

The type of vector and promoters to be employed should be selected, in part, depending on the organism and cell type to be affected. In the case of ex vivo stem cell therapy for human patients, a vector and promoter that are capable of transfection and expression in human cells should be selected.

In certain embodiments, retroviruses from which the retroviral plasmid vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, Rous sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. A retroviral plasmid vector may be employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, R-2, R-AM, PA12, T19-14.times., VT-19-17-H2, RCRE, RCRIP, GP+E-86, GP+envAml2, and DAN cell lines as described in Miller, Human Gene Therapy 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. A producer cell line generates infectious retroviral vector particles which include polynucleotide encoding a polypeptide of the present invention. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express a polypeptide of the present invention.

In certain embodiments, cells are engineered using an adeno-associated virus (AAV). AAVs are naturally occurring defective viruses that require helper viruses to produce infectious particles (Muzyczka, N., Curr. Topics in Microbiol. Immunol. 158:97 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate, but space for exogenous DNA is limited to about 4.5 kb. Methods for producing and using such AAVs are known in the art. See, for example, U.S. Pat. Nos. 5,139,941, 5,173,414, 5,354,678, 5,436,146, 5,474,935, 5,478,745, and 5,589,377. For example, an AAV vector may include all the sequences necessary for DNA replication, encapsidation, and host-cell integration. The recombinant AAV vector may be transfected into packaging cells which are infected with a helper virus, using any standard technique, including lipofection, electroporation, calcium phosphate precipitation, etc. Appropriate helper viruses include adenoviruses, cytomegaloviruses, vaccinia viruses, or herpes viruses. Once the packaging cells are transfected and infected, they will produce infectious AAV

viral particles which contain the polynucleotide construct. These viral particles are then used to transduce eukaryotic cells.

Essentially any method for introducing a nucleic acid construct into cells may be employed. Physical methods of introducing nucleic acids include injection of a solution containing the construct, bombardment by particles covered by the construct, soaking a cell, tissue sample or organism in a solution of the nucleic acid, or electroporation of cell membranes in the presence of the construct. A viral construct packaged into a viral particle may be used to accomplish both efficient introduction of an expression construct into the cell and transcription of the encoded shRNA. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical mediated transport, such as calcium phosphate, and the like. Thus the shRNA-encoding nucleic acid construct may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or otherwise increase inhibition of the target gene.

Cells to be transfected may be essentially any type of cell for implantation into in a subject. The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands. After transfection, stem cells may be administered as stem cells to a subject, or cultured to form further differentiated stem cells (e.g., embryonic stem cells cultured to form neural, hematopoietic or pancreatic stem cells) or cultured to form differentiated cells.

Stem cells may be stem cells recently obtained from a donor, and in certain preferred embodiments, the stem cells are autologous stem cells. Stem cells may also be from an established stem cell line that is propagated in vitro. Suitable stem

cells include embryonic stems and adult stem cells, whether totipotent, pluripotent, multipotent or of lesser developmental capacity. Stem cells are preferably derived from mammals, such as rodents (e.g. mouse or rat), primates (e.g. monkeys, chimpanzees or humans), pigs, and ruminants (e.g. cows, sheep and goats). Examples of mouse embryonic stem cells include: the JM1 ES cell line described in M. Qiu et al., Genes Dev **9**, 2523 (1995), and the ROSA line described in G. Friedrich, P. Soriano, Genes Dev **5**, 1513 (1991), and mouse ES cells described in US Patent No. 6,190,910. Many other mouse ES lines are available from Jackson Laboratories (Bar Harbor, Maine). Examples of human embryonic stem cells include those available through the following suppliers: Arcos Bioscience, Inc., Foster City, California, CyThera, Inc., San Diego, California, BresaGen, Inc., Athens, Georgia, ES Cell International, Melbourne, Australia, Geron Corporation, Menlo Park, California, Göteborg University, Göteborg, Sweden, Karolinska Institute, Stockholm, Sweden, Maria Biotech Co. Ltd. – Maria Infertility Hospital Medical Institute, Seoul, Korea, MizMedi Hospital – Seoul National University, Seoul, Korea, National Centre for Biological Sciences/ Tata Institute of Fundamental Research, Bangalore, India, Pochon CHA University, Seoul, Korea, Reliance Life Sciences, Mumbai, India, Technion University, Haifa, Israel, University of California, San Francisco, California, and Wisconsin Alumni Research Foundation, Madison, Wisconsin. In addition, examples of embryonic stem cells are described in the following U.S. patents and published patent applications: 6,245,566; 6,200,806; 6,090,622; 6,331,406; 6,090,622; 5,843,780; 20020045259; 20020068045. In preferred embodiments, the human ES cells are selected from the list of approved cell lines provided by the National Institutes of Health and accessible at http://escr.nih.gov. Examples of human adult stem cells include those described in the following U.S. patents and patent applications: 5,486,359; 5,766,948; 5,789,246; 5,914,108; 5,928,947; 5,958,767; 5,968,829; 6,129,911; 6,184,035; 6,242,252; 6,265,175; 6,387,367; 20020016002; 20020076400; 20020098584; and, for example, in the PCT application WO 0111011. In certain embodiments, a suitable stem cell may be derived from a cell fusion or dedifferentiation process, such as described in the following US patent application:

20020090722, and in the following PCT applications: WO200238741, WO0151611, WO9963061, WO9607732.

In some preferred embodiments, a stem cell should be compliant with good tissue practice guidelines set for the by the U.S. Food and Drug Administration (FDA) or equivalent regulatory agency in another country. Methods to develop such a cells may include donor testing, and avoidance of exposure to non-human cells and products during derivation of the stem cells.

In certain preferred embodiments, stem cells may be hematopoietic or mesenchymal stem cells, such as stem cell populations dervied from adult human bone marrow. Recent studies suggest that marrow-derived hematopoietic (HSCs) and mesenchymal stem cells (MSCs), which are readily isolated, have a broader differentiation potential than previously recognized. Many purified HSCs not only give rise to all cells in blood, but can also develop into cells normally derived from endoderm, like hepatocytes (Krause et al., 2001, *Cell* **105:** 369-77; Lagasse et al., 2000 *Nat Med* **6:** 1229-34). In at least one report (Lagasse et al, 2000 *Nat Med* **6:** 1229-34), the possibility of somatic cell fusion was ruled out. MSCs appear to be similarly multipotent, producing progeny that can, for example, express neural cell markers (Pittenger et al., 1999 *Science* **284:** 143-7; Zhao et al., 2002 *Exp Neurol* **174:** 11-20).

In certain embodiments, stem cells are derived from an autologous source or an HLA-type matched source. For example, HSCs may be obtained from the bone marrow of a subject in need of ex vivo cell therapy and cultured by a method described herein to generate an autologous cell compositions. Other sources of stem cells are easily obtained from a subject, such as stem cells from muscle tissue, stem cells from skin (dermis or epidermis) and stem cells from fat. Stem cell compositions may also be derived from banked stem cell sources, such as banked amniotic epithelial stem cells or banked umbilical cord blood cells.

Stem cells may also be crude or fractionated bone marrow-derived cells ("BMDCs"). BMDCs may be obtained from any stage of development of the donor individual, including prenatal (e.g., embryonic or fetal), infant (e.g., from birth to approximately three years of age in humans), child (e.g.. from about three years of

age to about 13 years of age in humans), adolescent (e.g., from about 13 years of age to about 18 years of age in humans), young adult (e.g., from about 18 years of age to about 35 years of age in humans), adult (from about 35 years of age to about 55 years of age in humans) or elderly (e.g., from about 55 years and beyond of age in humans).

In some embodiments, the BMDCs are transfected and administered as unfractionated bone marrow. Bone marrow may be fractionated to enrich for certain BMDCs prior to administration. Methods of fractionation are well known in the art, and generally involve both positive selection (i.e., retention of cells based on a particular property) and negative selection (i. e., elimination of cells based on a particular property). As will be apparent to one of skill in the art, the particular properties (e.g., surface markers) that are used for positive and negative selection will depend on the species of the donor bone marrow-derived cells.

When the donor bone marrow-derived cells are human, there are a variety of methods for fractionating bone marrow and enriching bone marrow-derived cells. A subpopulation of BMDCs includes cells, such as certain hematopoietic stem cells that express CD34, and/or Thy-1. Depending on the cell population to be obtained, negative selection methods that remove or reduce cells expressing CD3,CDIO,CDllb,CD14,CD16,CD15,CD16,CD19,CD20,CD32,CD45, CD45R/B220, Ly6G, and/or TER- 1 19 may be employed. When the donor BMDCs are not autologous, it is preferred that negative selection be performed on the cell preparation to reduce or eliminate differentiated T cells, thereby reducing the risk of graft versus host disease.

Cells will generally derive from verterbrates, particularly mammals. Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human.

Invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative generae of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris,

Trichuris, Trichostrongylus, Tflichonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconerriella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinerna). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

As will be apparent to one of skill in the art, it may be desirable to subject the recipient to an ablative regimen prior to administration of the shRNA transfected cells. Ablative regimens may involve the use of gamma radiation and/or cytotoxic chemotherapy to reduce or eliminate endogenous stem cells, such as hematopoietic stem cells and precursors. A wide variety of ablative regimens using chemotherapeutic agents are known in the art, including the use of cyclophosphamide as a single agent (50 mg/kg q day x 4), cyclophosphamide plus busulfan and the DACE protocol (4 mg decadron, 750 mg/m2 Ara-C, 50 mg/in 2carboplatin, 50 mg/m2 etoposide, q 12h x 4 IV). Additionally, gamma radiation may be used (e.g. 0.8 to 1.5 kGy, midline doses) alone or in combination with chemotherapeutic agents. In accordance with standard practice in the art, when chemotherapeutic agents are administered, it is preferred that the be administered via an intravenous catheter or central venous catheter to avoid adverse affects at the injection site(s).

4. Illustrative Uses

A. Methods of Treatment

In certain aspects, the invention provides methods of treating a disorder in a subject by introducing cells comprising a shRNA expression construct. In accordance with the methods disclosed herein, the shRNA may be reliably expressed in vivo in a variety of cell types. In certain embodiments the cells are administered in order to treat a condition. There are a variety of mechanisms by which shRNA expressing cells may be useful for treating a condition. For example, a condition may be caused in part by a population of cells expressing an undesirable gene. These cells may be ablated and replaced with administered cells comprising shRNA-that decreases expression of the undesirable gene; alternatively, the diseased cells

may be competed away by the administered cells, without need for ablation. As another example, a condition may be caused by a deficiency in a secreted factor. Amelioration of such a disorder may be achieved by administering cells expressing a shRNA that indirectly stimulates production of the secreted factor, e.g., by inhibiting expression of an inhibitor.

A shRNA may be targeted to essentially any gene, the decreased expression of which may be helpful in treating a condition. The target gene participate in a disease process in the subject. The target gene may encode a host protein that is co-opted by a virus during viral infection, such as a cell surface receptor to which a virus binds while infecting a cell. HIV binds to several cell surface receptors, including CD4 and CXCR5. The introduction of HSCs or other T cell precursors carrying an shRNA directed to an HIV receptor or coreceptor is expected to create a pool of resistant T cells, thereby ameliorating the severity of the HIV infection. Similar principles apply to other viral infections.

Immune rejection is mediated by recognition of foreign Major Histocompatibility Complexes. Where heterologous cells are to be administered to a subject, the cells may be transfected with shRNAs that target any MHC components that are likely to be recognized by the host immune system.

In many embodiments, the shRNA transfected cells will achieve beneficial results by partially or wholly replacing a population of diseased cells in the subject. The transfected cells may autologous cells derived from cells of the subject, but carrying a shRNA that confers beneficial effects.

B. Screening Assays

One utility of the present invention is as a method of identifying gene function in an organism, especially higher eukaryotes, comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceuticals, understanding normal and pathological events

associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for mammalian genomes, can be coupled with the invention to determine gene function in a cell or in a whole organism. The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which the dsRNA construct can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell or organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96 well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process.

In an exemplary embodiment, the subject invention provides an arrayed library of RNAi constructs. The array may be in the form of solutions, such as multi-well plates, or may be "printed" on solid substrates upon which cells can be grown. To illustrate, solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in

each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity.

In certain aspects, the invention provides methods for evaluating gene function in vivo. A cell containing an shRNA expression construct designed to decrease expression of a target gene may be introduced into an animal and a phenotype may be assessed to determine the effect of the decreased gene expression. An entire animal may be generated from cells (e.g., ES cells) containing an shRNA expression construct designed to decrease expression of a target gene. A phenotype of the transgenic animal may be assessed.

The animal may be essentially any experimentally tractable animal, such as a non-human primate, a rodent (e.g., a mouse), a lagomorph (e.g., a rabbit), a canid (e.g. a domestic dog), a feline (e.g., a domestic cat). In general, animals with complete or near complete genome projects are preferred.

A phenotype to be assessed may be essentially anything of interest. Quantitating the tendency of a stem cell to contribute to a particular tissue or tumor is a powerful method for identifying target genes that participate in stem cell differentiation and in tumorigenic and tumor maintenance processes. Phenotypes that have relevance to a disease state may be observed, such as susceptibility to a viral, bacterial or other infection, insulin production or glucose homeostasis, muscle function, neural regeneration, production of one or more metabolites, behavior patterns, inflammation, production of autoantibodies, obesity, etc.

A panel of shRNAs that affect target gene expression by varying degrees may be used, and phenotypes may be assessed. In particular, it may be useful to measure any correlation between the degree of gene expression decrease and a particular phenotype.

A heterogeneous pool of shRNA constructs may be introduced into cells, and these cells may be introduced into an animal. In an embodiment of this type of experiment, the cells will be subjected to a selective pressure and then it will be possible to identify which shRNAs confer resistance or sensitivity to the selective pressure. The selective pressure may be quite subtle or unintentional, for example, mere engraftment of transfected HSCs may be a selective pressure, with some

shRNAs interfering with engraftment and others promoting engraftment. Development and differentiation may be viewed as a "selective pressure", with some shRNAs modulating the tendency of certain stem cells to differentiate into different subsets of progeny. Treatment with a chemotherapeutic agent may be used as selective pressure, as described below. The heterogeneous pool of shRNAs may be obtained from a library, and in certain preferred embodiments, the library is a barcoded library, permitting rapid identification of shRNA species.

In certain aspects, the invention provides methods for identifying genes that affect the sensitivity of tumor cells to a chemotherapeutic agent. The molecular mechanisms that underlie chemoresistance in human cancers remain largely unknown. While various anticancer agents clearly have different mechanisms of action, most ultimately either interfere with DNA synthesis or produce DNA damage. This, in turn, triggers cellular checkpoints that either arrest cell proliferation to allow repair or provoke permanent exit from the cell cycle by apoptosis or senescence.

In certain embodiments, a method comprises introducing into a subject a transfected stem cell comprising a nucleic acid construct encoding an shRNA, wherein the shRNA is complementary to at least a portion of a target gene, wherein the transfected stem cell exhibits decreased expression of the target gene, and wherein the transfected stem cell gives rise to a transfected tumor cell in vivo. For example, the stem cell may be derived from an animal that has a genetic predisposition to tumorigenesis, such as an oncogene over-expressing animal (e.g. Eμ-myc mice) or a tumor suppressor knockout (e.g., p53 -/- animal). Alternatively, an animal comprising the stem cells may be exposed to carcinogenic conditions such that tumors comprising cells derived from the stem cells are generated. An animal having tumors may be treated with a chemotherapeutic or other anti-tumor regimen, and the effect of this regimen on cells expressing the shRNA may be evaluated. An shRNA that is overrepresented following anti-tumor therapy is likely to be targeted against a gene that confers sensitivity. An shRNA that is underrepresented following anti-tumor therapy is likely to be targeted against a gene that confers resistance. An shRNA that is underrepresented may be developed for use as a co-

therapeutic to be co-administered with the chemotherapeutic agent in question and suppress resistance.

Overrepresentation and underrepresentation are generally comparative terms, and determination of these parameters will generally involve comparison to a control or benchmark. A comparison may simply be to the same animal prior to chemotherapy administration. A comparison may also be to a control subject that has not received the chemotherapeutic agent. A comparison may be to an average of multiple other shRNA trials. Any control need not be contemporaneous with the experiment, although the protocol should be substantially the same.

This technique may be performed on individual shRNAs (see e.g., BIM shRNA, in the Examples below). The technique may also be adopted for highly parallel screening. For example, a method may comprise introducing into a subject a plurality of transfected stem cells, wherein each transfected stem cell comprises a nucleic acid construct comprising a representative shRNA of an shRNA library, and wherein a representative shRNA of an shRNA library is complementary to at least a portion of a representative target gene, wherein a plurality of the transfected stem cells exhibits decreased expression of a representative target gene, and wherein a plurality of the transfected stem cells gives rise to transfected tumor cells in vivo. Notably, it is not necessary or expected that every shRNA is different or that every transfected cell will become part of a tumor. Once tumors have been generated, a chemotherapeutic or other anti-tumor regimen may be administered, and the overrepresentation or underrepresentation of shRNA species may be evaluated. In certain preferred embodiments, each representative shRNA is associated with a distinguishable tag that permits rapid identification of each shRNA. For example, shRNAs may be obtained from a shRNA library that is barcoded.

Certain methods described herein take advantage of the fact that large numbers of cancer cells (e.g., lymphoma cells) can be isolated from affected mice and transplanted into syngeneic, immunocompetent recipients to create a lymphoma that is virtually indistinguishable from the spontaneous disease. This allows in vitro manipulation of tumor cells to create potentially chemoresistant variants that can be analyzed in vivo. In certain exemplary embodiments, the invention exploits

advantages of the Eμ-myc system to undertake an unbiased search for genetic alterations that can confer resistance to chemotherapeutics, such as the widely used alkylating agent, CTX.

The following is an outline of an example of a screen to identify genes that confer resistance to CTX using an unbiased, genetic approach. An overview of the screen is diagrammed in Fig 19. Populations of isolated lymphoma cells from the Eμ-myc mouse receive pools of sequence verified shRNAs that specifically target murine genes. Engineered cells are introduced into immunocompetent, syngeneic recipient animals. Upon the appearance of tumors, the animals are be treated with CTX. In each case, the time of remission is measured, and, upon relapse, the animals undergo a second round of treatment. After two rounds of therapy, the shRNA resident in resistant populations are identified and transferred into fresh populations of lymphoma cells, which are transplanted into naïve animals. After the appropriate number of selection cycles, individual shRNAs that are capable of conferring drug resistance are obtained.

C. Barcoding Methods

In certain embodiments, an expression construct that transcribes an RNAi species, e.g., a dsRNA or hairpin RNA, can include a barcode sequence. For those embodiments in which the RNAi constructs are provided as a variegated library for generating different RNAi species against a variety of different target sequence, each member (e.g., each unique target sequence) of the library can include a distinct barcode sequence such that that member of the library can be later identified if isolated individually or as part of an enriched population of RNAi constructs.

For example, two methods for determining the identity of the barcode sequence are by chemical cleavage, as disclosed by Maxim and Gilbert (1977), and by chain extension using ddNTPs, as disclosed by Sanger et al. (1977). In other embodiments, the sequence can be obtained by techniques utilizing capillary gel electrophoresis or mass spectroscopy. See, for example, U.S. Patent 5,003,059.

Alternatively, another method for determining the identity of a barcode sequence is to individually synthesize probes representing each possible sequence for each character position of a barcode sequence set. Thus, the entire set would

comprise every possible sequence within the barcode sequence portion or some smaller portion of the set. By various deconvolution techniques, the identity of the probes which specifically anneal to the barcode sequence sequences can be determined. An exemplary procedure would be to synthesize one or more sets of nucleic acid probes for detecting barcode sequence sequences simultaneously on a solid support. Preferred examples of a solid support include a plastic, a ceramic, a metal, a resin, a gel, and a membrane. A more preferred embodiment comprises a two-dimensional or three-dimensional matrix, such as a gel, with multiple probe binding sites, such as a hybridization chip as described by Pevzner et al. (J. Biomol. Struc. & Dyn. 9:399-410, 1991), and by Maskos and Southern (Nuc. Acids Res. 20:1679-84, 1992).

Hybridization chips can be used to construct very large probe arrays which are subsequently hybridized with a target nucleic acid. Analysis of the hybridization pattern of the chip provides an immediate fingerprint identification of the barcode sequence sequence. Patterns can be manually or computer analyzed, but it is clear that positional sequencing by hybridization lends itself to computer analysis and automation. Algorithms and software have been developed for sequence reconstruction which are applicable to the methods described herein (Drmanac et al., (1992) Electrophoresis 13:566-73; P. A. Pevzner, J. Biomol. Struc. & Dyn. 7:63-73, 1989).

For example, the identity of the barcode sequence sequence can be determined by annealing a solution of test sample nucleic acid including one or more barcode sequence sequences to a fixed array of character detection oligonucleotides (barcode sequence probes), where each column in the array preferably codes for one character of the barcode sequence. Each fixed oligonucleotide has a nucleotide base sequence that is complementary to the nucleotide base sequence of a single character. Either the test sample nucleic acid or the fixed oligonucleotides can be labeled in such a fashion to permit read-out upon hybridization, e.g., by radioactive labeling or chemiluminescent labeling. Test nucleic acid can be labeled, for example, by using PCR to amplify the identification region of a DNA pool under test with PCR primers that are radioactive or chemiluminescent. Preferred detectable labels include a radioisotope, a stable isotope, an enzyme, a fluorescent chemical, a

luminescent chemical, a chromatic chemical, a metal, an electric charge, or a spatial structure. There are many procedures whereby one of ordinary skill can incorporate detectable label into a nucleic acid.

For example, enzymes used in molecular biology will incorporate radioisotope labeled substrate into nucleic acid. These include polymerases, kinases, and transferases. The labeling isotope is preferably, ^{32}P, ^{35}S, ^{14}C, or ^{125}I.

Other, more advanced methods of detection include evanescent wave detection of surface plasmon resonance of thin metal film labels such as gold, by, for example, the BIAcore sensor sold by Pharmacia, or other suitable biosensors. An exemplary plasmon resonance technique utilizes a glass slide having a first side on which is a thin metal film (known in the art as a sensor chip), a prism, a source of monochromatic and polarized light, a photodetector array, and an analyte channel that directs a medium suspected of containing an analyte, in this case a barcode sequence-containing nucleic acid, to the exposed surface of the metal film. A face of the prism is separated from the second side of the glass slide (the side opposite the metal film) by a thin film of refractive index matching fluid. Light from the light source is directed through the prism, the film of refractive index matching fluid, and the glass slide so as to strike the metal film at an angle at which total internal reflection of the light results, and an evanescent field is therefore caused to extend from the prism into the metal film. This evanescent field can couple to an electromagnetic surface wave (a surface plasmon) at the metal film, causing surface plasmon resonance. When an array of barcode sequence probes are attached to the sensor chip, the pattern of annealing to barcode sequence sequences produces a detectable pattern of surface plasmon resonance on the chip.

The pattern of annealing, e.g., of selective hybriziation, of the labeled test DNA to the oligonucleotide array or the test DNA to the labeled oligonucleotide array permits the barcode sequence present in the original DNA clone to be directly read out. The detection array can include redundant oligonucleotides to provide integrated error checking.

In general, the hybridization will be carried out under conditions wherein there is little background (non-specific) hybridization, e.g., the background level is

at least one order of magnitude less than specific binding, and even more preferably, at least two, three or four orders of magnitude less.

Additionally, the array can contain oligonucleotides that are known not to match any barcode sequence in the library as a negative control, and/or oligonucleotides that are known to match all barcode sequences, e.g., primer flanking sequence, as a positive control.

5. Cell Delivery Systems

In certain embodiments, the invention provides a composition formulated for administration to a patient, such as a human or veterinary patient. A composition so formulated may comprise a stem cell comprising a nucleic acid construct encoding an shRNA designed to decrease the expression of a target gene. A composition may also comprise a pharmaceutically acceptable excipient. Essentially any suitable cell may be used, included cells selected from among those disclosed herein. Transfected cells may also be used in the manufacture of a medicament for the treatment of subjects. Examples of pharmaceutically acceptable excipients include matrices, scaffolds or other substrates to which cells may attach (optionally formed as solid or hollow beads, tubes, or membranes), as well as reagents that are useful in facilitating administration (e.g. buffers and salts), preserving the cells (e.g. chelators such as sorbates, EDTA, EGTA, or quaternary amines or other antibiotics), or promoting engraftment.

Cells may be encapsulated in a membrane or in a microcapsule. Cells may be placed in microcapsules composed of alginate or polyacrylates. (Lim et al. (1980) *Science* **210**:908; O'Shea et al. (1984) *Biochim. Biochys. Acta.* **840**:133; Sugamori et al. (1989) *Trans. Am. Soc. Artif. Intern. Organs* **35**:791; Levesque et al. (1992) *Endocrinology* **130**:644; and Lim et al. (1992) *Transplantation* **53**:1180). Additional methods for encapsulating cells are known in the art. (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) *Expt. Neurobiol.* **110**:39-44; Jaeger et al. (1990) *Prog. Brain Res.* **82**:41-46; and Aebischer et al. (1991) *J. Biomech. Eng.* **113**:178-183, U.S. Patent No. 4,391,909; U.S. Patent No. 4,353,888; Sugamori et al. (1989) *Trans. Am. Artif.*

Intern. Organs **35**:791-799; Sefton et al. (1987) *Biotehnol. Bioeng.* **29**:1135-1143; and Aebischer et al. (1991) *Biomaterials* **12**:50-55).

The site of implantation of insulin-producing cell compositions may be selected by one of skill in the art depending on the type of cell and the therapeutic objective. Exemplary implantation sites include intravenous or intraarterial administration, administration to the liver (via portal vein injection), the peritoneal cavity, the kidney capsule or the bone marrow.

EXAMPLES

Example 1: Stable introduction of shRNA-transfected cells into mice

In this Example, Applicants demonstrate the introduction of an RNA interference construct into stem cells and the stable maintenance of an RNA interference-derived phenotype in vivo after cell implantation. The test system is the Eμ-myc transgenic mouse system established by Applicants; these mice overexpress the myc gene in B cell lineages and generate lymphoma-like tumors. Features of the Eμ-myc mouse model include: (i) Eμ-myc lymphomas recapitulate typical genetic and pathological features of human Non-Hodgkin's lymphomas; (ii) tumors arise with relatively short latency and high penetrance; (iii) tumor burden can be easily monitored by lymph-node palpation or blood smears; (iv) lymphomas are detectable long before the animal dies; (v) large numbers of pure tumor cells can be isolated from enlarged lymph-nodes for biochemical studies; (vi) therapy is performed in immunocompetent mice; and (vii) lymphoma cells can be cultured and transplanted into syngeneic, non-transgenic recipient mice. In addition, Applicants have developed methods for manipulating the genotype of Eμ-myc lymphomas, allowing the creation of tumors with defined genetic lesions and an assessment of the relationship of these to treatment responses. This also allows 'tagging' of tumor cells with fluorescent proteins and monitoring of tumor burden by in vivo imaging in live mice. Furthermore, Applicants have previously demonstrated that Myc-initiated lymphomas can be generated with different secondary lesions by (i) intercrossing to genetically engineered mice, (ii) rapidly transferring retroviral genes into established lymphomas, or (iii) retrovirally infecting hematopoietic stem cells

prior to their propagation in myeloablated recipient mice. These different approaches can be combined in a way that lymphomas with multiple genotypes are rapidly produced. See, e.g., Schmitt, C.A., et al., A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell, 2002. 109(3): p. 335-46; Schmitt, C.A., C.T. Rosenthal, and S.W. Lowe, Genetic analysis of chemoresistance in primary murine lymphomas. Nat Med, 2000. 6(9): p. 1029-35; Schmitt, C.A, Fridman, J.S., Yang, M., Baranov, E., Hoffman, R.M., and Lowe, S.W. Dissecting p53 tumor suppressor functions in vivo. Cancer Cell 2002. 1: p. 289-98.

Tumor cells which express exogenous genes may be generated by harvesting hematopoietic stem cells from Eμ-myc transgenic fetal livers and introducing various constructs using recombinant retroviruses. These cells are transplanted into multiple lethally irradiated recipient animals by tail vein injection. Applicants have shown that these mice develop B-cell tumors in an equivalent time frame to their non-transplanted counterparts (Schmitt et al., Cancer Cell 1:289-98 (2002)).

Applicants have previously published that Eμ-myc mice, which are p53-/-, develop tumors at an accelerated rate (Schmitt et al., Genes Dev. 13:2670-77 (1999)). Here applicants show that various p53 shRNAs introduced into a p53 +/+ background can recapitulate the p53 -/- phenotype and accelerate tumor formation to varying degrees. Of note, applicants have shown that the acuteness of the phenotype is dependent on the hairpin applicants use. In essence, applicants can generate a panel of hairpins which result in a gradient of activity; fully functional, 75% functional, 50% functional and so forth. This type of panel is quite useful in analyzing a specific gene's contribution to the biology of a condition, such as a tumor. The biological activity of these shRNAs is further demonstrated by the lack of loss of heterozygosity (LOH) in p53 +/- Eμ-myc tumors expressing the short hairpins compared to 100% LOH in control tumors. Applicants have also been able to isolate cells from shRNA expressing tumors and re-transplant them into syngenic mice. The arising tumors continue to suppress p53 and are as aggressive as their p53-/- counterparts.

Materials and Methods

Generation of p53 shRNA retroviruses-p53 hairpin oligos were designed using designated software found at http://katahdin.cshl.org:9331/RNAi/. The hairpins described in this application have the following sequence: p53-1-

AAAAAGGTCTAAGTGGAGCCCTTCGAGTGTTAGAAGCTTGTGACACTCG GAGGGCTTCACTTGGGCCCGGTGTTTCGTCCTTTCCACAA AND p53-2-

AAAAAAAACATCCGACTGCGACTCCTCCATAGCAGCAAGCTTCCTGCCA TGGAGGAGTCACAGTCGGATATCGGTGTTTCGTCCTTTCCACAA. To generate hairpin sequences downstream of U6 promoter, PCR reactions were run using a pGEM U6 promotor template (provided by Greg Hannon), the p53 hairpin primers and a CACC-SP6 reverse primer with the following sequence: CACCGATTTAGGTGACACTATAG. The PCR conditions were the following: 100ng pGEM U6 plasmid, 1μM p53 hairpin primer, 1μM SP6, 1x Perkin-Elmer PCR reaction buffer (with 15mM MgCl2), 4% DMSO, .25mM dNTPs and 5 Units of taq DNA polymerase. Reactions were run for 1x 95 degrees for 5 minutes, 30 cycles of 95 degrees 30", 55 degrees 30" and 72 degrees 1'. PCR products were then blunted by incubating at 72 degrees for 10 minutes in the presence of 2 units of pfu DNA polymerase. PCR products were cloned directly into a pENTR/TOPO-D vector (Invitrogen), using the company specifications. Plasmids containing the PCR product were cut with EcoRV and gel extracted. The cut plasmid was placed into a "Gateway™" reaction (Invitrogen) reaction with a retroviral vector containing a "Gateway™ destination cassette" and the Gateway™ BP clonase enzyme mix. The reaction was performed as specified in the Gateway™ BP clonase enzyme product literature. Retroviral vectors containing destination cassettes were created as follows: pBabe Puro was cut with Nhe1 and a linear reading frame cassette A (Gibco/Brl) fragment was blunt-end ligated into the cut vector in the 3' LTR. MSCV puro (Clontech) was cut with Hpal and a linear reading frame cassette A was blunt-end ligated into the cut vector upstream of the PGK promoter.

Retroviral Infection of Stem Cells- Stem cells were isolated from the fetal livers of EμMyc transgenic mice as described (Schmitt et al, Cancer Cell 1(2):289-98). Genotyping for the presence of the EμMyc transgene was done as described.

Retroviral infection was performed using vectors p53-A, p53-B and p53-C as described (Schmitt et al., Cancer Cell. 2002 (3):289-98).

Tumor Analysis- Tumor burden was monitored externally by lymph node palpation. The presence of the hairpin DNA in tumors was confirmed by performing the same PCR reaction described above, replacing the pGEM U6 template with 100ng of tumor DNA. H&E staining of lymph nodes, lung and spleen in recipient animals was performed to confirm the presence of a pathology consistent with B-cell lymphoma. TUNEL assays were performed to determine the level of in-tumor apoptosis.

LOH Analysis- Retroviral infection of p53+/- stem cells was performed using vectors p53-A, p53-B and p53-C as described (Schmitt et al, Cancer Cell, 1(3):289-98 (2002)). The genotype of the recipient stem cells and the resulting DNA was performed as described.

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Example 2: Germline transmission of RNAi in mice

MicroRNA molecules (miRNAs) are small, noncoding RNA molecules that have been found in a diverse array of eukaryotes, including mammals. miRNA precursors share a characteristic secondary structure, forming short 'hairpin' RNAs. Genetic and biochemical studies have indicated that miRNAs are processed to their mature forms by Dicer, an RNAse III family nuclease, and function through RNA-mediated interference (RNAi) and related pathways to regulate the expression of target genes (Hannon 2002, Nature 418: 244-251; Pasquinelli et al. 2002, Annu. Rev. Cell. Dev. Biol. 18: 495-513). Recently, applicants and others have remodeled miRNAs to permit experimental manipulation of gene expression in mammalian cells and have dubbed these synthetic silencing triggers 'short hairpin RNAs' (shRNAs) (Paddison et al. 2002, Cancer Cell 2: 17-23). Silencing by shRNAs requires the RNAi machinery and correlates with the production of small interfering RNAs (siRNAs), which are a signature of RNAi.

Expression of shRNAs can elicit either transient or stable silencing, depending upon whether the expression cassette is integrated into the genome of the recipient cultured cell (Paddison et al. 2002, Cancer Cell 2: 17-23). shRNA expression vectors also induce gene silencing in adult mice following transient delivery (Lewis et al. 2002,.Nat. Genet. 32: 107-108; McCaffrey et al. 2002, Nature 418: 38-39). However, for shRNAs to be a viable genetic tool in mice, stable manipulation of gene expression is essential. As shown in Example 1, Applicants have demonstrated long-term suppression of gene expression *in vivo* following retroviral delivery of shRNA-expression cassettes to hematopoietic stem cells. Here Applicants demonstrated a methodology by which shRNA-expression cassettes that are passed through the mouse germline can enforce heritable gene silencing.

Applicants began by taking standard transgenesis approaches (Gordon et al. 1993, Methods Enzymol. 225: 747-771) using shRNAs directed against a variety of targets with expected phenotypes, including the genes encoding tyrosinase (albino), myosin VIIa (shaker), Bmp-5 (crinkled ears), Hox a-10 (limb defects), homogentisate 1,2,-dioxygenase (urine turns black upon exposure to air), Hairless (hair loss) and melanocortin 1 receptor (yellow). Three constructs per gene were

linearized and injected into pronuclei to produce transgenic founder animals. Although applicants noted the presence of the transgene in some animals, virtually none showed a distinct or reproducible phenotype that was expected for a hypomorphic allele of the targeted gene.

Therefore, applicants decided to take another approach: verifying the presence of the shRNA and its activity toward a target gene in cultured embryonic stem (ES) cells and then asking whether those cells retained suppression in a chimeric animal *in vivo*. Applicants also planned to test whether such cells could pass a functional RNAi-inducing construct through the mouse germline. For these studies, applicants chose to examine a novel gene, *Neil1*, which is proposed to have a role in DNA repair. Oxidative damage accounts for 10,000 DNA lesions per cell per day in humans and is thought to contribute to carcinogenesis, aging and tissue damage following ischemia (Ames et al. 1993, Proc. Natl. Acad. Sci. USA 90: 7915-7922; Jackson et al. 2001, Mutat. Res. 477: 7-21). Oxidative DNA damage includes abasic sites, strand breaks and at least 20 oxidized bases, many of which are cytotoxic or pro-mutagenic (Dizdaroglu et al. 2002, Free Radic. Biol. Med. 32: 1102-1115). DNA *N*-glycosylases initiate the base excision repair pathway by recognizing specific bases in DNA and cleaving the sugar base bond to release the damaged base (David et al. 1998, Chem. Rev. 98: 1221-1262).

The *Neil* genes are a newly discovered family of mammalian DNA *N*-glycosylases related to the Fpg/Nei family of proteins from *Escherichia coli* (Hazra et al. 2002, Proc. Natl. Acad. Sci. USA 99: 3523-3528; Bandaru et al. 2002, DNA Repair 1: 517-529). Neil1 recognizes and removes a wide spectrum of oxidized pyrimidines and ring-opened purines from DNA, including thymine glycol (Tg), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formidopyrimidine (FapyA). Tg, FapyG and FapyA are among the most prevalent oxidized bases produced by ionizing radiation (Dizdaroglu et al. 2002, Free Radic. Biol. Med. 32: 1102-1115) and can block replicative DNA polymerases, which can, in turn, cause cell death (Asagoshi et al. 2002, J. Biol. Chem. 277: 14589-14597; Clark et al. 1989, Biochemistry 28: 775-779).

The Nth1 and Ogg1 glycosylases each remove subsets of oxidized DNA bases that overlap with substrates of Neil1 (Nishimura 2002, Free Radic. Biol. Med. 32: 813-821; Asagoshi et al. 2000, Biochemistry 39: 11389-11398; Dizdaroglu et al. 1999, Biochemistry 38: 243-246). However, mice with null mutations in either *Nth1* (Ocampo et al. 2002, Mol. Cell. Biol. 22: 6111-6121; Takao et al. 2002, EMBO J. 21: 3486-3493) or *Ogg1* (Klungland et al. 1999, Proc. Natl. Acad. Sci. USA 96: 13300-13305; Minowa et al. 2000, Proc. Natl. Acad. Sci. USA 97: 4156-4161) are viable, raising the possibility that Neil1 activity tempers the loss of Nth1 or Ogg1. Recently, a residual Tg-DNA glycosylase activity in *Nth1*$^{-/-}$ mice has been identified as Neil1 (Takao et al. 2002, J. Biol. Chem. 277: 42205-42213).

Applicants constructed a single shRNA expression vector targeting a sequence near the 5' end of the *Neil1* coding region. This vector was introduced into mouse embryonic stem cells by electroporation, and individual stable integrants were tested for expression of the Neil1 protein (see the weblink: http://www.cshl.edu/public/SCIENCE/hannon.html for detailed procedures). The majority of cell lines showed an ~80% reduction in Neil1 protein, which correlated with a similar change in levels of *Neil1* mRNA. These cells showed an approximately two-fold increase in their sensitivity to ionizing radiation, consistent with a role for Neil1 in DNA repair. Two independent ES cell lines were injected into BL/6 blastocysts, and several high-percentage chimeras were obtained. These chimeras were out-crossed, and germline transmission of the shRNA-expression construct was noted in numerous F_1 progeny (13/27 for one line and 12/26 for the other).

To determine whether the silencing of *Neil1* that had been observed in ES cells was transmitted faithfully, applicants examined *Neil1* mRNA and protein levels. Both were reduced by approximately the same extent that had been observed in the engineered ES cells (Figs. 9, 10). Consistent with this having occurred through the RNAi pathway, applicants detected the presence of siRNAs corresponding to the shRNA sequence in F_1 animals that carry the shRNA expression vector but not in those that lack the vector (Fig. 10b).

The aforementioned data demonstrate that shRNAs can be used to create germline transgenic mice in which RNAi has silenced a target gene. These observations open the door to using of RNAi as a complement to standard knock-out methodologies and provide a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. Coupled with activator-dependent U6 promoters, the use of shRNAs will ultimately provide methods for tissue-specific, inducible and reversible suppression of gene expression in mice.

Example 3: shRNA Modification of Stem Cells: Bim and Puma

Example 1, above, describes the use of p53 shRNA constructs to reduce p53 levels in hematopoietic stem cells. This reduction in p53 levels, in conjunction with Myc overexpression, was sufficient to produce tumor phenotypes in reconstituted recipient animals. Here, Applicants demonstrate the broad applicability of this technology for reducing gene expression in stem cells by targeting two additional putative tumor suppressors: Bim and Puma.

Bim and Puma shRNA constructs were created as described for the shp53 constructs. The primers used to create Bim shRNAs were:

mBim-1 -
AAAAAAATCACACTCAGAACTCACACCAGAAGGCTCAAGCTTCAACCTT
CTGATGTAAGTTCTGAGTGTGACGGTGTTTCGTCCTTTCCACAA

mBim-2 -
AAAAAAAAGAGTAGTCTTCAGCCTCGCAGTAATCACAAGCTTCTGATTA
CCGCGAGGCTGAAGACCACCCTCGGTGTTTCGTCCTTTCCACAA

mBim-3-
AAAAAAGAGATAGGGACCCCAAGCCTGAGCTGGAGCAAGCTTCCCCCA
GCTCAGGCCTGGGGCCCCTACCTCGGTGTTTCGTCCTTTCCACAA

The primers used to create Puma shRNAs were:

mPUMA-1 -
AAAAAAGAGAGCCGCCCTCCTAGCATGCGCAGGCCCAAGCTTCGGCCCG
CGCACGCCAGGAGGGCAGCTCTCGGTGTTTCGTCCTTTCCACAA

mPUMA-2 -
AAAAAAGGGACTCCAAGATCCCTGAGTAAGAGGAGCAAGCTTCCTCCCC
TTACCCAGGGATCCTGGAGCCCCGGTGTTTCGTCCTTTCCACAA

mPUMA-3 -
AAAAAAGGGAGGGCTAAGGACCGTCCGAGCACGAGCAAGCTTCCCCGC
GCCCGGACGGTCCTCAGCCCTCCCGGTGTTTCGTCCTTTCCACAA

After PCR reactions using a U6 template (see Example 1), the resulting U6 shRNA PCR products were transferred into both MSCV Puro and MSCV Puro-IRES-GFP retroviral constructs. Virus generated from MSCV Puro Bim shRNA and MSCV Puro-IRES-GFP Puma shRNA constructs was used to infect Em-Myc hematopoietic stem cells. The infected stem cells were then used to reconstitute the hematopoietic system of irradiated recipient mice.

Mice receiving MSCV Puro Bim shRNA and MSCV Puro-IRES-GFP Puma shRNA developed lymphomas at a significantly higher penetrance and shorter onset time than mice receiving control vector (Figure 11A). RT-PCR of total RNA was performed on tumors from mice receiving control or MSCV Puro Bim shRNA vectors, using the following primers:

mBim5'-Xho1 CCGCTCGAGGCCACCATGGCCAAGCAACCTTCTGATG

mBim3'-EcoRI CCGGAATTCTCAATGCCTTCTCCATACCAGACG

Tumors arising in mice receiving MSCV Puro Bim shRNA virus showed a nearly complete reduction in all Bim splice forms, while control tumors showed significant amount of Bim RNA (Figure 11B). Western blots were performed on tumors from control vector and MSCV Puro-IRES-GFP Puma shRNA mice, using an Anti-Puma antibody (Axxora, LLC). Tumors arising in mice receiving MSCV Puro-IRES-GFP Puma shRNA virus showed a significant reduction in Puma expression relative to control-infected tumors (Figure 11C).

These results establish that 1) stable RNAi in stem cells is possible for a wide variety of target genes, 2) shRNA constructs can produce stable phenotypes in recipient cells and 3) these constructs specifically repress their proposed targets.

Example 4: Modulating Chemotherapeutic Resistance in Stem Cells and Tumor Cells using Stable RNAi

Bim plays a well-established role in antagonizing Bcl-2 function, and Bcl-2 overexpression has previously been shown to mediate chemotherapeutic resistance *in vivo*. To examine whether gene suppression by RNAi could affect treatment response, as well as tumor formation, we examined the response of tumors created with MSCV Puro Bim shRNAs to chemotherapy. Control and Bim shRNA tumors were treated with 10mg/kg adriamycin and monitored for tumor-free survival by regular palpation and blood smears (see Schmitt et al., Cancer Cell 2002; Cell). Bim shRNA tumors showed a significant decrease in tumor free survival and time to death relative to control tumors (Figure 12). Thus, stem cells engineered to express shRNAs can yield tumors with distinct chemotherapeutic sensitivities.

Given this ability of shRNAs to modulate tumor treatment response in tumors arising from shRNA-modified stem cells, we wanted to determine whether stable RNAi could modulate chemotherapeutic response acutely in mature tumors. Previous work from our group has shown that Em-Myc ARF-/- tumors are sensitive to adriamycin treatment (Schmitt et al, Cell 2002). To determine whether stable RNAi could alter the treatment response of chemosensitive tumors, we infected Em-Myc ARF-/- tumors with either a control vector or MSCV Puro-IRES-GFP Bim shRNA (Schmitt et al. Nature Med 2000). Following infection, the number of infected tumor cells was assayed by FACs analysis, and equal percentages of control and shBIM-infected tumors cells were injected into WT recipient animals (Figure 13). Tumors arising in recipient animals were treated with 10mg/kg adriamycin. Relapsed tumors were assessed for GFP content by FACs analysis (Figures 13 and 14). In the case of control-infected tumors, relapsing tumors were GFP-negative, suggesting that the presence of the vector conferred no selective advantage on these tumor cells. However, tumors relapsing after shBIM stable infection were invariably GFP-positive, indicating that the tumor cells expressing the Bim hairpin had a selective advantage after treatment. This data establishes that shRNAs can modulate tumor sensitivity, and that shRNAs can be used to screen for mediators of drug sensitivity.

These data demonstrate the feasibility of a a global strategy to identify modifiers of drug action in vivo. Specifically, if an shRNA is enriched during treatment responses (as occurs for shBIM), then inactivation of the target gene confers a survival advantage during treatment. As such, the nature of such shRNAs will provide insight into the molecular basis of drug action as well as to potential mechanisms of drug resistance. In contrast, if an shRNA is depleted, then inactivation of the target gene sensitizes the cell to killing in the presence of the drug. The nature of these depleted shRNAs will provide insights into possible targets or pathways that would work in combination with the drug. Of note, while studies may be performed on individual shRNAs, the development of 'bar-coded' shRNA libraries (described herein) will greatly facilitate this effort. Finally, while these experiments use mouse tumors, similar studies may be performed on human tumor cells in xenograft settings.

Example 5: SIN shRNA Vectors

We have generated Self-INactivating retroviruses that express shRNAs. These viruses, based on the Clontech pQCXIX self-inactivating retrovirus contain an inactive 5' LTR following viral insertion, resulting in the absence of long viral transcript expression. Experiments with p53 shRNAs (as described in Example 1) show that these vectors produce significantly better suppression of p53 in mouse embryonic fibroblasts than MSCV vectors expressing the same shRNA (Figure 15A and B). This provides the first direct evidence that the SIN vectors may be more effective than standard vectors.

Example 6: Characterization of Germline Transgenic Mice

As described above, Applicants have developed methods for generating mice expressing shRNAs in the germline. Applicants have further characterized p53 shRNA expressing mice generated using lentiviral transduction.

A lentiviral vector encoding our "p53C" shRNA was used to infect embryos and produce mice expressing a functional hairpin. Further characterization of these

mice shows that of 10 pups born, 3 founder mice (#3, #8, and #10) were confirmed to harbor the shRNA construct by GFP fluorescence, PCR and Southern blot. Genomic DNA from each animal was digested with Pst 1, Southern blotted and hybridized with a GFP + WRE probe as per protocol in Lois et al. 2002. Southern blots of tail DNA indicate that each founder animal has have a single proviral insertion. This is important, as it will minimize complications associated with multiple gene copy numbers and providing a simple method of tracking transgenic animals.

Western analysis of p53 in the dermal fibroblasts of the transgenic founder mice has revealed that p53 protein levels are significantly reduced, even in the presence of the DNA damaging agent adriamycin (Figure 16). In contrast, the non-transgenic littermate controls (#1 and #2), as expected, show robust p53 activation in response to adriamycin treatment. Thus, we are able to achieve stable RNAi in the whole animal.

To confirm the functionality of the p53 hairpin, we performed colony-formation assays using the dermal fibroblasts isolated from the transgenic founders and non-transgenic littermates. In this assay, p53 deficiency results in a greatly enhanced ability of untransformed cells to form colonies when plated at clonogenic density. Data shown in Figure 17 indicate the ability of the fibroblasts from the transgenic founder mice to form significantly more colonies compared to fibroblasts from the non-transgenic littermate controls. Consistently, cells from the non-transgenic animals underwent replicative senescence at approximately passage 7 (as assessed by growth rate, morphology, and Senescence-Associated β-galactosidase staining). In contrast, no senescent cells have been detected in cells obtained from the transgenic founders (currently at passage 12).

Finally, Applicants have demonstrated the ability of the founders to transmit the transgene to their progeny. Transgenic founder mouse #10 produced 2 separate litters of pups, several of which were positive for GFP and by PCR of regions of the vector.

Example 7: Generation of shRNA Libraries and Highly Parallel Screening

Applicants have constructed a partial genome-wide library of RNAi inducing constructs that will eventually target every gene in the human genome. Applicants have targeted ~8,500 genes with approximately 23,000 sequence-verified shRNAs. Each is carried in a validated, MSCV-derived vector that is immediately useful for stable suppression. However, Applicants have also designed the vectors to have the capability of moving the inserts to other vectors via a recombination strategy that occurs *in vivo* following bacterial mating. Applicants can easily move any insert from the library into the lentiviral backbone that is used for transgenesis experiments described above.

Additionally, each component of the library is tagged with an individual barcode. These allow one to follow the changes in the numbers of cells representing individual clones in the library (in a mixed population) using oligonucleotide microarrays. Applicants have prepared such arrays and are now testing the possibility of doing large-scale synthetic lethality screens using this strategy.

In the one version of the library, the distribution of shRNAs was skewed to enrich for sequences that matched also the mouse homolog of a given gene. This has resulted in our accumulating about 6,000 mouse shRNA constructs so far. A second generation library is a specifically targeted mouse library. Applicants have selected approximately 1,200 genes, which have each been targeted with 5 shRNA sequences. Genes in this set were selected based upon their cancer relevance and were hand-curated

Each shRNA expression cassette in the mouse and human RNAi libraries is associated with a unique 60 nucleotide barcode. This permits the use of population genetics as an approach to the search for both positively and negatively selected epigenetic lesions in screens of the libraries. For example, imagine a search for shRNAs that enhance the sensitivity of cells to doxorubicin or a targeted therapeutic. Cells would be infected with the library such that each of the 20,000 shRNAs is represented by 100-1000 infected cells. This population is treated with the drug at a relatively low concentration, e.g. EC10. By comparing untreated and treated populations, we might find shRNAs that enhance sensitivity to a low concentration of drug, since these would be selectively lost from the population. The ability to

conduct such a screen depends upon parallel analysis of individual cell populations expressing shRNA constructs. One could also examine the behavior of pure homogeneous populations of cells bearing individual shRNAs in 96 or 384 well plates. However, the availability of barcoded vectors lets us track the frequency of individual shRNA clones in a mixed population, allowing highly parallel assays to be conducted in vitro or in vivo.

Barcode arrays corresponding to the 22,600 hairpins in the human shRNA library have been synthesized. These have been validated by self-self hybridizations using both DNA from the E. coli library and DNA where the barcodes have been amplified from the genomic DNA of library-infected 3T3 cells. Quality control test have demonstrated that the arrays perform well, with 2,600 negative controls appearing as negatives, and with the barcodes known to be represented in the population giving positive signals. There are a small number of false positives (<1%) that may be eliminated by further optimization of hybridization conditions. Examination of a comparative intensity plot shows most spots reporting consistently in Cy3 and Cy5 labeled material. All of the spots falling off of the diagonal can be accounted for by an easily recognizable anomaly in the hybridization signal (Figure 18).

Example 8: Certain Transgenic Animal Protocols

a) ShRNA Transgenic Mice: Isolation of shRNA ES-cell lines. Standard ES-cell techniques are employed. A 129S6/SvEvTac TC1 cell line was obtained from Harvard Medical School (Boston, MA, Dr. P. Leder). The ES-cells are routinely maintained between passage 11-15 by culture on irradiated MEF-feeder cells in ES-media further supplemented with LIF-containing conditioned media. 20 μg of linearized plasmid DNA is electroporated into ~107 ES-cells. The electroporated cells are plated onto gelatinized plates and cultured in ES-media supplemented with LIF-containing conditioned media. After two days Geneticin (Roche) is added to an active concentration of 300μg/ml. The cells are cultured for an additional ten days to allow colony formation. From each selection ~50 colonies with undifferentiated ES-cell morphology are cloned by trypsinization and 96-well plates. After 4 further days of growth the cells are cryopreserved in situ on two of the 96-well plates to

preserve them at early passage. The third replicate cultures are then grown further by passage to 12-well then 6-well plates. At that point separate aliquots of cells are cryopreserved, and lysed for either DNA, RNA or protein isolation to determine transgene presence and knockdown of target gene expression.

Chimeric mouse production. Blastocysts are isolated from 8 super-ovulated E3.5d pregnant C57Bl/6 mice and cultured in ES-cell media. ES-cells are trypsinized to single-cells and washed in ES-media. Five to ten ES-cells are injected into each blastocysts. The injected ES-cells are then transferred to the uterus of 2.5d pseudo-pregnant CD-1 foster females in batches of 8-10. For each cell line 50 blastocysts are injected. Chimeric pups are born 17 days post-injection. The degree of ES-cell contribution in chimeric pups is estimated from the degree of agouti coat color. In our experience the TC1 cell line, although XY in karyotype, frequently generates gametes in both male and female chimeras. Thus 4-6 high percentage chimeras of either sex are bred to C57Bl/6 females to determine the degree of germline contribution of the ES-cells in each chimera through coat color genetics of the F1 pups. Germline–competent chimeras are then bred to 129/SvEvTac mice (from Taconic Farms) to maintain the shRNA transgene on an inbred background. The presence of the shRNA transgene in F1 pups is determined by PCR of tail biopsy DNA.

b) Lentiviral Transgenics

shRNA expressing lentiviruses are resuspended at 106 ifu/ml in M2 media, aliquoted in 10µl portions and stored at –80 degrees. For sub-zonal injection of fertilized mouse eggs the viral suspension is thawed and centrifuged briefly in a table-top microcentrifuge. Five microliters of suspension is then placed under mineral oil on a glass coverslip mounted in an injection chamber. Also on the cover slip is placed a 5 µl drop of CZB medium supplemented with 1µg/ml Cytochalasin B. Fertilized eggs are incubated for 10 minutes in the CZB-cytochalasin prior to injection. For injection the viral suspensiom is picked up into a micropipette with a 2-5 µM aperture. The injection pipet is transferred to drop with the eggs. Positive pressure of 0.5-2 PSI is applied to the viral suspension to promote a slow steady outward flow. Each egg is then picked up with a holding pipet and the injection

pipet is allowed to puncture the zona pellucida of the egg. A slight swelling of the zona indicates flow of the viral suspension into the peri-vitteline space. Each egg is injected similarly. Following injection the eggs are transferred to a dish of M2 media and then sequentially through four 200µl drops of M2 media to dilute the cytochalasin B. Finally the embryos are transferred to a 37 degree incubator for culture in M16 media. All of the injection pipets, injection chambers, etc are rinsed in 70% Ethanol:1% SDS to inactivate lentiviruses.

Injected embryos are transferred to the oviduct of pseudo-pregnant CD1 mice. Potentially transgenic pups are born 19 days later. At 1 week of age tail biopsies are performed for DNA extraction. The tail DNA is screened by PCR to identify transgenic pups with genomic lentiviral insertions. Positive pups will be further screened by southern blot DNA analysis to determine copy number of the insertions.

Example 9. Generation of chimeric mice using RCAS/TVA

Applicants have generated a vector system that will allow tissue specific expression of shRNAs in vivo. This approach involves infecting cells expressing an avian viral receptor under the control of a ubiquitous or tissue-specific promoter in vivo. Applicants have modified the RCAS vectors to optimally express our RNAi haripins in mice and generated vectors that express shRNAs targeting mouse p53. As a proof of the system, Applicants generated virus from these constructs and used it to infect MEFs stably expressing the avian viral receptor. The functionality of these hairpins was confirmed by immunofluorescence, using p53 antibodies, which showed a dramatic reduction in p53 levels in cells infected with RCAS p53 shRNA constructs (infected cells are GFP-positive) (Figure 21). This apparent loss of p53 was confirmed in a classic p53 functional assay. Specifically, MEFs infected with RCAS p53 grew well when plated at low density, while control cells were unable to produce colonies (Figure 22). This data establishes that shRNAs can effectively target genes when expressed from RCAS retroviral vectors.

Example 10: Generation of ES cells expressing shRNAs.

This examples describes a system for creating genetically defined RNAi "epi-alleles" in mice using Cre-mediated recombination to stably integrate a single RNAi expression cassette into a single locus in the mouse genome. This technique will minimize clonal variation due to random integration events seen in other studies and should allow for the efficient creation of "epi-allelic" series of RNAi constructs, as well as an inducible RNAi system. Applicants have adapted a system developed for chromosomal engineering in mice to mediate the integration of a single short hairpin RNA (shRNA) expression cassette in mouse ES cells. This strategy relies on the ability to integrate a "donor" plasmid, containing a shRNA expression construct, into an "acceptor" locus through the transient expression of Cre recombinase (Figure 23). This system is designed so that proper recombinants can be selected for, through the reconstitution of the mini-HPRT gene and a drug resistance gene (eg, puromycin). Additionally, both the donor and acceptor constructs express coat color gene markers, either Agouti or Tyrosinase, which can be used to score chimeric mice.

This system has been tested in *hprtΔ* ES cells at the D4Mit190 locus. By co-transfecting either a Cre expression vector and the shRNA donor plasmid or the donor plasmid alone, 100% of HPRT reconstituted ES cell colonies (ie HATr colonies) (90 of 90) contain correctly integrated donor plasmids (as scored by genomic PCR). Importantly no HATr colonies were observed in the absence of Cre recombinase, suggesting that this scheme is highly effective at inducing site-specific integration in ES cells.

To test the effectiveness of this approach at evoking gene silencing in ES cells, Applicants integrated an shRNA cassette expressing a hairpin targeting Firefly luciferase. Individual HATr clones were isolated and transiently transfected with plasmids expressing Firefly luciferase (i.e., the target gene) and Renilla luciferase (i.e., a transfection control which is not targeted). The results, shown in Figure 24, demonstrate that clones harboring the Firefly shRNA can potently suppress luciferase activity, (approximately 5-fold relative to control cells).

Example 11: Reversible RNAi in vivo

Applicants have generated a novel retroviral vector (MSCV CreER/loxP U6shRNA PIG; Figure 25A) containing all the genetic components required to reversibly inhibit gene function by RNAi. This vector is based on the MSCV U6shRNA GFP vector (see above).

To facilitate conditional deletion of the provirus, a loxP site is engineered into the NheI restriction site of the MSCV 3' LTR, resulting in a floxed provirus upon integration (Figure 25A). In addition, Applicants placed a cassette encoding the $CreER^{T2}$ fusion protein upstream of the U6shRNA cassette, under the control of the viral 5' LTR promoter. In normal cells, $CreER^{T2}$ is cytoplasmic and inactive, however addition of tamoxifen activates the recombinase activity of the fusion protein.

Using the p53C shRNA, Applicants have shown that each component of the vector appears to be functional. MEFs infected with MSCV CreER/loxP U6p53C PIG virus show stable suppression of p53 expression by Western blot (Figure 25B). Therefore the CreER fusion protein and loxP sites do not interfere with shRNA production. Addition of 0.5 μM 4-hydroxytamoxifen (4OHT) to cultured cells infected with MSCV CreER/loxP U6p53C PIG virus results in deletion of the provirus from the genome, as measured by Southern blot using a probe that hybridizes to the GFP cassette in the provirus (Figure 26A). As expected, 4OHT treatment and excision of the provirus also leads to loss of GFP expression, as measured by Western blot (Figure 26B) or FACS (Figure 26C). Fluorescence microscopy also shows loss of GFP flourescence upon 4OHT treatment of cultured cells infected with MSCV CreER/loxP U6p53C PIG virus. These results demonstrate that the CreER fusion protein encoded by the provirus can effectively excise the provirus itself. Importantly, 4OHT treatment does not appear to affect growth of uninfected cultured cells, and excision of the provirus occurs after only 24 hours of 4OHT treatment. This self-excising strategy has three major benefits: (1) the timing of Cre activation can be controlled; (2) long-term Cre toxicity is avoided; and (3) all infected cells (producing shRNAs) have the intrinsic potential to delete

the provirus. Each of these factors are important when adapting this approach to in vivo tumor models.

Applicants have examined the effects of reversing RNAi-mediated knockdown of p53 expression in cultured primary cells. Initial observations indicate that excision of the p53C shRNA-producing cassette in late passage murine embryonic fibroblasts causes substantial cell death (Figure 27). Applicants have also initiated in vivo "reversible tumorigenesis" experiments using the Eμ-myc lymphoma model. Systemic tamoxifen treatment has proven effective in other animal model systems and it should be able to effectively reverse RNAi-mediated suppression of gene expression in established tumor cells in vivo. The MSCV CreER/loxP self-excising viral vector should allow us to test proof of principle for "hit and run" gene therapy approaches based on RNAi or gene overexpression.

A second generation vector is shown in Figure 28. This vector has several modifications that may make it more effective. First, the retroviral vector is a contains a self-inactiating (SIN) LTR such that, upon provirus integration, there is no transcription from the 5' LTR. This modification should increase the effectiveness of shRNA mediated silencing, as shown in 'RNAi stem cells 1; Figure 28. Second, the cre-ER IRES GFP cassette is placed downstream of the strong CMV promoter, which will increase the expression of both components, allowing better excision of the provirus upon tamoxifen addition and better visualization of GFP in vitro and in vivo. Note also that other recombination systems and regulatable recombinases could be used as well.

This vector or similar ones (e.g. based on lentivirus technology) will have broad applications for in vitro and in vivo use. First, one can envision manipulating stem cells ex vivo with an shRNA in a reversible way (i.e. 'hit and run' gene therapy). This might be advantageous in settings where transient gene suppression is desirable or, in the event that some hairpins direct stable gene silencing (as can occur in some species), removal of the vector leaving the suppression intact. In fact results indicate that excision of a p53 targeted shRNA construct from a cell does not result in recovery of p53 expression (Figure 29). This indicates that an epigenetic change is occurring, resulting in a permanent or at least heritable inhibition of p53

expression even in the absence of a shRNA construct. Cells may therefore be transfected with a shRNA construct ex vivo to initiate downregulation, the construct removed, and the cells administered to a patient. In this manner, a patient receives genetically unmodified cells that have an engineered gene expression pattern. Second, for the construction of animal models of human disease, one envisions inactivating a gene using an excisable shRNA, allowing a phenotype to be produced, and then reversing the mutations to see whether the phenotype is rescued.

One example would be to inactivate a tumor suppressor gene, allow a cancer to form in an animal, add tamoxifen to excise the provirus (and shRNA) and then determine whether the cancer progresses upon re-expression of the tumor suppressor. This will show whether the tumor suppressor gene is required for tumor maintenance of the tumor, and would determine whether the pathway might be suitable for therapeutic intervention (i.e. if the tumor suppressor is required for tumor maintenance the pathway would be a good target). A second, broader, application would be to generate animal models of recessive human disorders using ES cells or some other stem cell type. Upon the appearance of a deleterious phenotype, tamoxifen can be administered to the animal, which is subsequently monitored for reversal of the deleterious phenotype. For example, one could produce a mouse model of muscular dystrophy or a neurodegerative disease by suppressing the causative gene, and then ask, at what point during the progression of the disease, the phenotype is reversible (in some settings the disease may have progressed beyond a point of no return). Such information would provide a guide as to when a disease can be corrected by pharmaceutical means or gene therapy.

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

EQUIVALENTS

While specific embodiments of the subject inventions are explicitly disclosed herein, the above specification is illustrative and not restrictive. Many variations of the inventions will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the inventions should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.